Synthesis and Evaluation of Tumor Cell Growth Inhibition of Methyl 3-Amino-6-[(hetero)arylethynyl]thieno[3,2-*b*]pyridine-2-carboxylates. Structure-Activity Relationships, Effects on the Cell Cycle and Apoptosis

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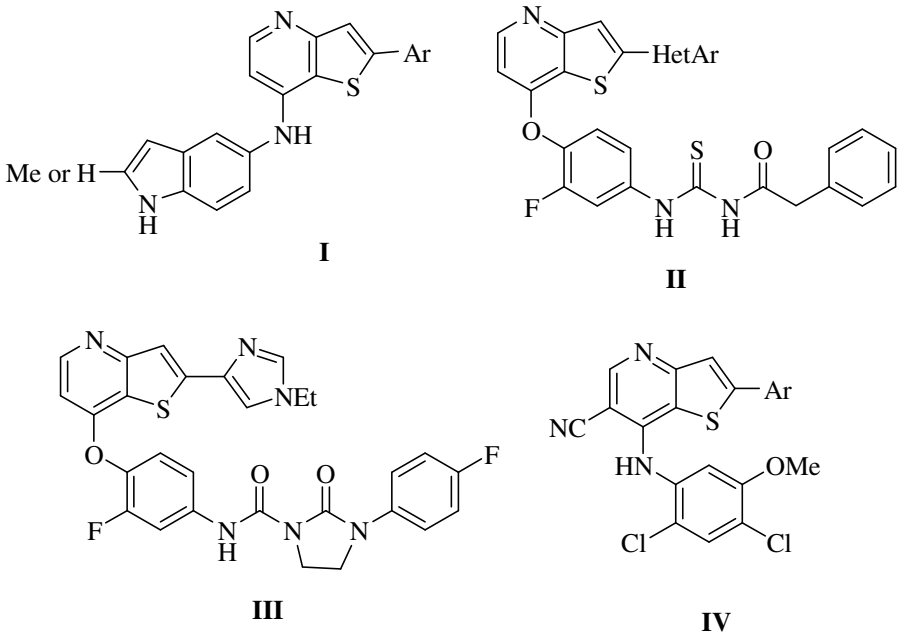
Abstract - The methyl 3-amino-6-bromothieno[3,2-*b*]pyridine-2-carboxylate, recently reported by some of us, was reacted in Sonogashira couplings with several (hetero)arylacetylenes. The growth inhibitory activity of the novel methyl 3-amino-6-[(hetero)arylethynyl]thieno[3,2-*b*]pyridine-2-carboxylates obtained was evaluated on three human tumor cell lines (MCF-7, NCI-H460, A375-C5). The *para*-methoxyphenyl and the *ortho* and *para*-aminophenyl derivatives were the most promising compounds, and their effects were further studied regarding alterations in the normal cell cycle distribution and induction of apoptosis in the NCI-H460 cell line. All three compounds altered cell cycle distribution and the *ortho*-aminophenyl derivative was further shown to induce apoptosis in the same cell line.

Keywords: Thieno[3,2-*b*]pyridines, Sonogashira coupling, Antitumoral activity, Cell Cycle, Apoptosis

1. Introduction

Recently several thieno[3,2-*b*]pyridines have shown important biological activities, namely antitumor and antiangiogenesis or dual activity, essentially by acting as inhibitors of tyrosine kinase receptors **I** [1], **II** [2] **III** [3], or nonreceptors (**IV**) [4] which have been implicated in the growth and progression of various human cancers and, therefore, have been crucial in the development of anticancer therapies.



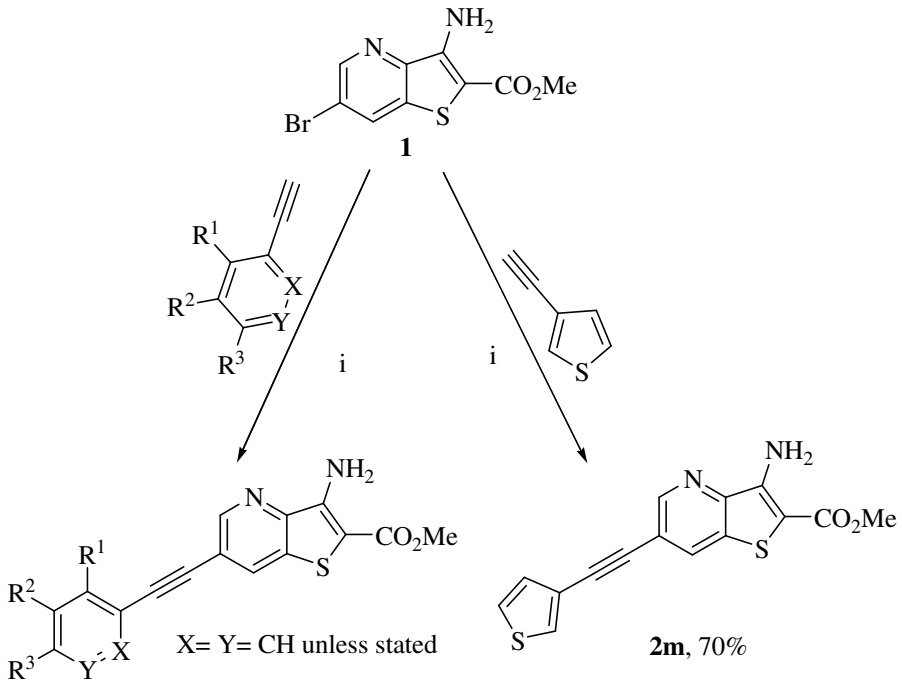
As we have recently prepared the methyl 3-amino-6-bromothieno[3,2-*b*]pyridine-2-carboxylate [5] it was decided to use it in the Pd/Cu-catalysed Sonogashira coupling [6] with several terminal (hetero)arylacetylenes in the synthesis of novel methyl 3-amino-6-[(hetero)arylethynyl]thieno[3,2-*b*]pyridine-2-carboxylates. Their potential as antitumor agents was evaluated through the *in vitro* growth inhibitory activity on three selected human tumor cell lines, MCF-7, NCI-H460, and A375-C5 and some structure-activity relationships (SARs) were established.

For the most promising compounds the effects on the cell cycle distribution and induction of apoptosis were studied, in order to investigate their possible mechanisms of action.

2. Results and discussion

2.1 Synthesis

The 6-bromothieno[3,2-*b*]pyridine **1** [5] was used as the thieno[3,2-*b*]pyridine component in the Pd/Cu catalyzed Sonogashira reaction [5-7] with several (hetero)arylacetylenes to obtain the novel methyl 3-amino-6-[(hetero)arylethynyl]thieno[3,2-*b*]pyridine-2-carboxylates **2a**-**m** in high to excellent yields using the conditions depicted in Scheme 1, already used by us in the synthesis of arylethynyl benzo[*b*]thiophene derivatives [7].



2a $R^1 = R^2 = R^3 = H$, 70%
2b $R^1 = OMe$, $R^2 = R^3 = H$, 80%
2c $R^2 = OMe$, $R^1 = R^3 = H$, 75%
2d $R^3 = OMe$, $R^1 = R^2 = H$, 70%
2e $R^1 = NH_2$, $R^2 = R^3 = H$, 70%
2f $R^2 = NH_2$, $R^1 = R^3 = H$, 60%
2g $R^3 = NH_2$, $R^1 = R^2 = H$, 75%
2h $R^3 = NMe_2$, $R^1 = R^2 = H$, 80%
2i $R^1 = F$, $R^2 = R^3 = H$, 75%
2j $R^3 = F$, $R^1 = R^2 = H$, 90%
2k $R^1 = R^2 = R^3 = H$, X = N,70%
2l $R^1 = R^2 = R^3 = H$, Y = N, 90%

Scheme 1. Synthesis of compounds **2a-2m**. Conditions: $PdCl_2(PPh_3)_2$ (5 mol%), CuI (3 mol%), NEt_3 (3 equiv.), DMF, 1.5 h, 100 ^oC.

2.2 Growth inhibition of human tumor cell lines

The *in vitro* growth inhibitory activity of all the compounds prepared was evaluated using three human tumor cell lines, representing different tumor types, namely breast adenocarcinoma (MCF-7), melanoma (A375-C5), and non-small cell lung cancer (NCI-H460), after a continuous exposure of 48 h. The results are summarized in Table 1.

Table 1- Growth inhibitory activity of the 6-(hetero)arylethynylthieno[3,2-*b*]pyridines **2** on the three human tumor cell lines in study.

Compounds	GI_{50} (μM)[a]		
	MCF-7	**A375-C5**	**NCI-H460**
2a	>150.0	>150.0	>150.0
2b	>47.3[b]	>47.3[b]	>47.3[b]
2c	33.4±4.7	>75.0[b]	49.0±4.7
2d	0.46±0.05	>37.5[b]	0.32±0.05
2e	4.1±0.7	3.1±2.1	5.7±3.0
2f	10.8±1.2	11.6±0.6	11.5±0.5
2g	2.6±1.4	4.8±0.4	8.9±0.9
2h	20.6±1.1	17.7±0.1	16.6±1.1
2i	>150	>150	>150
2j	29.0±1.2	48.4±1.6	24.0±2.0
2k	37.4±4.1	13.9±1.9	34.9±5.4
2l	>150	>150	>150
2m	27.7±2.7	29.6±0.2	24.8±0.5

[a]The lowest concentrations causing 50% of cell growth inhibition (GI_{50}) after a continuous exposure of 48 h, expressed as means ± SEM of three independent experiments performed in duplicate. [b] Due to the low solubility of the compound in DMSO the maximum concentration tested was lower than 150 μM. Doxorubicin was used as positive control (GI50: MCF-7 = 43.3 ± 2.6 nM; A375-C5 = 130.2 ± 10.1 nM; NCI-H460 = 35.6 ± 1.6 nM).

From the analysis of Table 1 it can be concluded that the *p*-methoxylated compound **2d** presents the lowest GI_{50} values (below 1.0 μM), with marked selectivity for MCF-7 and NCI-H460 cell lines. The aminated compounds **2e**-**2g** were also effective at inhibiting cell growth, with GI_{50} values between 2.6-11.6 μM for the three cell lines, the best being the *o*- and *p*-aminophenyl compounds **2e** and **2g**.

The methoxylated compounds **2b** and **2c** were not very potent at inhibiting cell growth. They are not very soluble, nevertheless for compound **2c** it was possible to determine the GI_{50} values for MCF-7 and NCI-H460 which were between 33.4 and 49.0 μM. The tendency for selective activity excluding the melanoma cell line (A375-C5), which is clear for compound **2d**, is already detectable in **2c**. The *p*-dimethylamino derivative **2h** presents higher GI_{50} values than the free amino compounds. Among the fluorinated compounds **2i** and **2j** only the latter shows moderate GI_{50} values (24.0-48.4 μM). Considering the fact that the unsubstituted phenylacetylene derivative **2a** did not show any activity at the highest tested concentration, the cell growth inhibitory activity presented by the compounds discussed above appears to be closely dependent on the substitution pattern.

The pyridine derivative **2k** shows some selectivity against A375-C5 cell line, with a GI_{50} value of 13.9 μM. The position of the C-C bond in the pyridine ring appears to be determinant for the cell growth inhibitory activity, since compound **2l** was not active at the highest concentration tested.

The thiophene derivative **2m** presents moderate growth inhibitory activity against the three cell lines in study (GI_{50} values between 24.8-29.6 μM).

2.3. Cell cycle analysis and detection of apoptosis

The most active compounds were chosen to be further investigated regarding their mechanism of action. The NCI-H460 cell line was incubated with the GI_{50} concentrations of compounds **2d**, **2e** and **2g** for 48 h and their effects on the normal cell cycle distribution and induction of apoptosis analyzed. The results show that all three compounds interfere with the normal cell cycle distribution of this cell line. Compound **2d** caused a small increase in the percentage of cells in G0/G1 and a reduction in the percentage of cells in the S-phase of the cell cycle (Table 2). A similar but more marked effect was observed upon incubation with compound **2g**. Compound **2e** induced an increase in the percentage of cells in G2/M phases of the cell cycle accompanied by a

decrease of cells in S-phase (Table 2). From these results it appears that compounds **2d** and **2g** induce a cell cycle arrest on phases G0/G1, whereas compound **2e** appears to cause a G2/M cell cycle arrest. These compounds were also shown to induce apoptosis, as determined by the annexin V-FICT/PI flow cytometry assay, particularly compound **2e** (Table 2). This observation was confirmed by the presence of a sub-G1 peak in the cell cycle profile analysis (indicative of DNA degradation, characteristic of apoptosis) upon incubation with compound **2e** (data not shown).

Table2. Cell cycle analysis (% of cells in phases G0/G1, S, G2/M) and % of NCI-H460 cells undergoing apoptosis after a 48 h treatment with compounds **2d**, **2e** and **2g** at their GI_{50} concentrations.

	G0/G1 (cells %)	**S** (cells %)	**G2/M** (cells %)	**Apoptosis** (cells %)
Control	57.1 ± 1.6	28.2 ± 1.4	14.7 ± 0.9	11.7 ± 1.6
DMSO	55.1 ± 1.0	28.8 ± 1.2	16.1 ± 0.9	12.2 ± 1.2
2d	59.7 ± 3.2	22.0 ± 1.7	18.3 ± 1.9	18.7 ± 2.8
2e	54.7 ± 2.7	23.1 ± 2.8	24.6 ± 2.4	25.3 ± 1.8[a]
2g	68.0 ± 0.7	18.6 ± 0.8	13.4 ± 0.6	17.0 ± 2.1

Untreated cells were used as the control and a solvent control (DMSO) was also included. Results are the mean ± standard error of three to six independent experiments performed in duplicate.
[a] Apoptosis induction by compound **2e** was statistically significantly when compared to the control (P <0.05).

According to these findings, the compounds are likely to present different mechanisms of action. The effect of compound **2e** in cell growth inhibition seems to be due to the induction of both apoptosis and cell cycle arrest in the G2/M phase (Table2). Compounds **2d** and **2g** did not cause such a marked induction of apoptosis (Table 2) and thus their growth inhibitory effects seem to be mainly due to cell cycle related events.

3. Conclusion

Novel methyl 3-amino-6-[(hetero)arylethynyl]thieno[3,2-*b*]pyridine-2-carboxylates were synthesized and tested for their *in vitro* growth inhibitory activity on three human tumor cell lines. All compounds were fully soluble at the GI_{50} concentration. Overall, the *para*-methoxyphenyl and the *ortho* and *para*-aminophenyl derivatives are the most

promising compounds, presenting very low GI_{50} values and therefore their effects on cell cycle distribution and induction of apoptosis were further investigated in the NCI-H460 cell line. Compounds **2d**, **2e** and **2g** had an effect on cell cycle distribution, with a G0/G1 arrest being detected for the *para*-methoxyphenyl and the *para*-aminophenyl derivatives and a G2/M arrest for the *ortho*-aminophenyl derivative. Additionally, the *ortho*-aminophenyl compound was found to significantly induce apoptosis at the GI_{50} concentration in the tested cell line. Hence, at least two mechanisms of action appear to be involved in the activity of this compound, cell cycle related events and induction of apoptosis. The obtained data suggest that the results are dependent on the compound substitution pattern, nevertheless it is important to bear in mind that the physical properties of the compounds, particularly cell permeability, may be influencing their relative activity.

4. Experimental

4.1 Synthesis

Melting points (°C) were determined in a SMP3 Stuart apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity Plus at 300 and 75.4 MHz, and on a Bruker Avance II at 400 and 100.6 MHz, respectively. Heteronuclear correlations, ^{1}H-^{13}C, HMQC or HMSQ and HMBC were performed to attribute some signals. MS (EI) and HRMS data were recorded by the mass spectrometry service of the University of Vigo, Spain. Elemental analysis was performed on a LECO CHNS 932 elemental analyser. The reactions were monitored by thin layer chromathography (TLC). Dry flash was performed on Macherey-Nagel silica gel 150-230 mesh. Petroleum ether refers to the boiling range 40-60 °C. Ether refers to diethylether.

4.1.1. General procedure for the synthesis of compounds 2a-2m:
Compound **1** (150 mg, 0.540 mmol), the (hetero)arylacetylene (1.1 equiv.), $PdCl_2(PPh_3)_2$ (5 mol%), CuI (3 mol%), and NEt_3 (3 equiv.) were added to dry DMF (2-3 mL) into a dry Schlenk tube, under argon, and the mixture was heated with stirring at 100 ºC for 1.5 h. After cooling, water (5 mL) and ethyl acetate (5 mL) were added and the phases were separated. The aqueous phase was then extracted with additional ethyl acetate (3 × 5 mL). The organic phases were collected, dried ($MgSO_4$), filtered, and, finally, removal of the solvent under reduced pressure gave a solid which was submitted to column chromatography using 50% ether/ petroleum ether as eluent. The solid obtained was washed with petroleum ether and fully characterized.

4.1.1.1. Methyl 3-amino-6-(2-phenylethynyl)thieno[3,2-*b*]pyridine-2-carboxylate (2a): Yellow solid (117 mg, 70%), m.p. 175-177 ºC. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.93 (3H, s, OMe), 6.24 (2H, br s, NH_2), 7.38-7.42 (3H, m, Ar-H), 7.56-7.60 (2H, m, Ar-H), 8.18 (1H, d, *J* = 2 Hz, Ar-H), 8.73 (1H, d, *J* = 2 Hz, Ar-H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ 51.75 (OMe), 86.07 (C), 93.88 (C), 100.84 (2-C), 119.11(2×CH), 122.29 (1'-C), 128.50 (2×CH), 129.02 (C), 131.74 (C), 133.49 (CH), 133.72 (C), 144.97 (3a-C), 147.30 (C), 148.73 (CH), 165.17 (C=O) ppm. MS (EI) m/z 308 (M^+, 100), 276 (66). HRMS M^+ calcd. for $C_{17}H_{12}N_2O_2S$ 308.0619; found 308.0624.

4.1.1.2. Methyl 3-amino-6-[2-(2-methoxyphenyl)ethynyl]thieno[3,2-*b*]pyridine-2-carboxylate (2b): Yellow solid (146 mg, 80%), m.p. 193-195 ºC. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.92 (3H, s, OMe), 3.95 (3H, s, OMe), 6.22 (2H br s, NH_2), 6.93-6.98 (2H, m, Ar-H), 7.34-7.39 (1H, m, Ar-H), 7.53 (1H, dd, *J* = 7.6 and 2 Hz, Ar-H), 8.20 (1H, d, *J* = 2 Hz, Ar-H), 8.75 (1H, d, *J* = 2 Hz, Ar-H) ppm. ^{13}C NMR ($CDCl_3$,

100.6 MHz) δ 51.71 (OMe), 55.83 (OMe), 90.06 (C), 90.45 (C), 100.68 (2-C), 110.74 (CH), 111.52 (C), 119.50 (C), 120.57 (CH), 130.57 (CH), 133.38 (CH), 133.65 (CH), 133.67 (C), 144.89 (C), 147.38 (C), 148.89 (CH), 160.16 (C), 165.21 (C=O) ppm. MS (EI) m/z 338 (M^+, 100), 306 (39). HRMS M^+ calcd. for $C_{18}H_{14}N_2O_3S$ 338.0725; found 338.0727.

41.1.3. Methyl 3-Amino-6-[2-(3-methoxyphenyl)ethynyl]thieno[3,2-*b*]piridine-2-carboxylate (2c): Yellow solid (137 mg, 75%), m.p. 177-179 ºC. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.85 (3H, s, OMe), 3.93 (3H, s, OMe), 6.23 (2H, br s, NH_2), 6.94-6.97 (1H, m, Ar-H), 7.09-7.11 (1H, m, Ar-H), 7.16-7.19 (1H, m, Ar-H), 7.30 (1H, app. t, *J* = 8 Hz, 5'-H), 8.19 (1H, d, *J* = 1.6 Hz, Ar-H), 8.73 (1H, d, *J* = 1.6 Hz, Ar-H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ 51.74 (OMe), 55.33 (OMe), 85.89 (C), 93.78 (C), 100.84 (2-C), 115.65 (CH), 116.48 (CH), 119.03 (C), 123.26 (C), 124.29 (CH), 129.58 (5'-CH), 133.49 (CH), 133.69 (C), 145.05 (C), 147.32 (C), 148.78 (CH), 159.42 (C), 165.18 (C=O) ppm. MS (EI) m/z 338 (M^+, 100), 306 (53). HRMS M^+ calcd. for $C_{18}H_{14}N_2O_3S$ 338.0725; found 338.0723.

4.1.1.4. Methyl 3-amino-6-[2-(4-methoxyphenyl)ethynyl]thieno[3,2-*b*]pyridine-2-carboxylate (2d): yellow solid (130 mg, 70 %), m.p. 190-192 ºC. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.85 (3H, s, OMe), 3.92 (3H, s, OMe), 6.25 (2H, broad s, NH_2), 6.92 (2H, d, J = 8.4 Hz, 3' and 5'-H), 7.52 (2H, d, J = 8,4 Hz, 2' and 6'-H), 8.16 (1H, d, J = 2 Hz, Ar-H), 8.71 (1H, d, J = 2 Hz, Ar-H) ppm. ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ 51.74 (OMe), 55.34 (OMe), 84.90 (C), 94.17 (C), 100.63 (2-C), 114.16 (3'and 5'-CH), 114.28 (C), 119.52 (C), 133.25 (CH), 133.29 (2' and 6'-CH), 133.78 (C), 144.56 (C), 147.27 (C), 148.56 (CH), 160.20 (C), 165.18 (C=O) ppm. MS (EI): m/z (%) 338 (M^+, 100), 306 (39). HRMS M^+ $C_{18}H_{14}N_2O_3S$: caldt. 338.0725; found 338.0724.

4.1.1.5. Methyl 3-amino-6-[2-(2-aminophenyl)ethynyl]thieno[3,2-*b*]pyridine-2-carboxylate (2e): Yellow solid (122 mg, 70%), m.p. 168-170 ºC. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 3.82 (3H, s, OMe), 5.68 (2H, br s, NH_2), 6.52-6.57 (1H, m, Ar-H), 6.73 (1H, br d, *J* = 9.2 Hz, Ar-H), 6.93 (2H, br s, 2'-NH_2), 7.08-7.14 (1H, m, Ar-H), 7.26 (1H, dd, *J* = 7.6 and 1.6 Hz, Ar-H), 8.63 (1H, d, *J* = 1.6 Hz, Ar-H), 8.85 (1H, d, *J* = 1.6 Hz, Ar-H) ppm. ^{13}C NMR (DMSO-d_6, 100.6 MHz) δ 51.57 (OMe), 90.88 (C), 91.60 (C), 97.94 (2-C), 104.38 (C), 114.03 (CH), 115.69 (CH), 119.02 (C), 130.46 (CH), 131.89 (CH), 132.97 (C), 133.50 (CH), 144.57 (C), 147.77 (C), 148.51 (CH), 150.15 (C), 164.40 (C=O) ppm. Elemental Anal. calcd for $C_{17}H_{13}N_3O_2S$ C, 63.14; H, 4.05; N, 12.99; S, 9.92%. Found C, 63.05; H, 4.05; N, 12.59; S, 9.48%.

4.1.1.6. Methyl 3-amino-6-[2-(3-aminophenyl)ethynyl]thieno[3,2-*b*]pyridine-2-carboxylate (2f): Yellow solid (105 mg, 60%), m.p. 203-205 ºC. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 3.82 (3H, s, OMe), 5.30 (2H, br s, NH_2), 6.64 (1H, ddd, J = 8.1 2.4 and 1.2 Hz, Ar-H), 6.73 (1H, dt, J = 7.6 and 1.2 Hz, Ar-H), 6.77 (1H, m, Ar-H), 6.92 (2H, br s, NH_2), 7.01 (1H, app. t, *J* = 8 Hz, 5'-H), 8.57 (1H, d, *J* = 2 Hz, Ar-H), 8.76 (1H, d, *J* = 2 Hz, Ar-H) ppm. ^{13}C NMR (DMSO-d_6, 100.6 MHz) δ 51.62 (OMe), 84.97 (C), 94.46 (C), 98.35 (2'-C), 115.17 (4'-CH), 116.14 (2'-CH), 118.42 (C), 118.94 (6'-CH), 121.68 (C), 129.32 (5'-CH), 133.10 (C), 133.92 (CH), 144.94 (C), 147.65 (C), 148.53 (CH), 148.91 (C), 164.40 (C=O) ppm. MS (EI) m/z 323 (M^+, 100), 291 (56). HRMS calcd. for $C_{17}H_{13}N_3O_2S$ M^+ 323.0728; found 323.0729.

4.1.1.7. Methyl 3-amino-6-[2-(4-aminophenyl)ethynyl]thieno[3,2-*b*]pyridine-2-carboxylate (2g): Yellow solid (130 mg, 75%), m.p. 211-213 ºC. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 3.81 (3H, s, OMe), 5.70 (2H, br s, NH_2), 6.57 (2H, d, *J* = 8.4 Hz, 3' and 5'-CH), 6.91 (2H, br s, NH_2), 7.25 (2H, d, *J* = 8.4 Hz, 2' and 6'-CH), 8.46 (1H, d, *J* = 2 Hz, Ar-H), 8.70 (1H, d, *J* = 2 Hz, Ar-H) ppm. ^{13}C NMR (DMSO-d_6, 75.4 MHz) δ 51.60 (OMe), 83.87 (C), 96.06 (C), 97.85 (2-C), 107.07 (C), 113.62 (3' and 5'-CH), 119.43 (C), 132.92 (2' and 6'-CH), 132.97 (CH), 133.22 (C), 144.33 (C), 147.77 (C), 148.29 (CH), 150.15 (C), 164.46 (C=O) ppm. MS (EI) m/z 323 (M^+, 100), 291 (51). HRMS calcd. for $C_{17}H_{13}N_3O_2S$ M^+ 323.0728; found 323.0727.

4.1.1.8. Methyl 3-amino-6-{2-[4-(dimethylamino)phenyl]ethynyl}thieno[3,2-*b*]pyridine-2-carboxylate (2h): Green solid (152 mg, 80%), m.p. 191-193 ºC. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.96 (6H, s, 2×Me), 3.81 (3H, s, OMe), 6.73 (2H, d, *J* = 8.7 Hz, 3' and 5'-CH), 6.92 (2H, br s, NH_2), 7.40 (2H, d, *J* = 8.7 Hz, 2' and 6'-CH), 8.49 (1H, d, *J* = 2 Hz, Ar-H), 8.72 (1H, d, J = 2 Hz, Ar-H) ppm. ^{13}C NMR (DMSO-d_6, 75.4 MHz) δ 39.59 (2×Me), 51.56 (OMe), 84.51 (C), 95.60 (C), 97.86 (2-C), 107.33 (1'C), 111.79 (3' and 5'-CH), 119.24 (C), 132.66 (2' and 6'-CH), 133.05 (CH), 133.16 (C), 144.36 (3a-C), 147.72 (C), 148.28 (CH), 150.44 (4'-C), 164.41 (C=O) ppm. MS (EI) m/z 351 (M^+, 100), 319 (33). HRMS calcd. for $C_{19}H_{17}N_3O_2S$ M^+ 351.1041; found 351.1042.

4.1.1.9. Methyl 3-amino-6-[2-(2-fluorophenyl)ethynyl]thieno[3,2-*b*]pyridine-2-carboxylate (2i): Yellow solid (132 mg, 75%), m.p. 174-176 ºC. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.93 (3H, s, OMe), 6.22 (2H, br s, NH_2), 7.14-7.18 (2H, m, Ar-H), 7.35-7.41 (1H, m, Ar-H), 7.53-7.58 (1H, m, Ar-H), 8.21 (1H, d, *J* = 2 Hz, Ar-H), 8.75 (1H, d, *J* = 2 Hz, Ar-H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ 51.75 (OMe), 87.10 (C), 91.03 (C), 101.00 (2-C), 111.06 (d, *J* = 16 Hz, C), 115.68 (d, *J* = 21 Hz, CH), 118.68 (C), 124,12 (d, *J* = 3 Hz, CH), 130.78 (d, *J* = 8 Hz, CH), 133.53 (d, *J* = 13 Hz, CH), 133.63 (CH), 145.29 (C), 147.31 (C), 148.77 (CH), 162.73 (d, *J* = 251 Hz, C-F), 165.16 (C=O) ppm. Elemental Analysis calcd. for $C_{17}H_{11}FN_2O_2S$ C, 62.57; H, 3.40; N, 8.58; S, 9.83%. Found C, 62.39; H, 3.43; N, 8.40; S, 9.43%.

4.1.1.10. Methyl 3-amino-6-[2-(4-fluorophenyl)ethynyl]thieno[3,2-*b*]pyridine-2-carboxylate (2j): Yellow solid (159 mg, 90%) m.p. 167-169 ºC. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.93 (3H, s, OMe), 6.23 (2H, br s, NH_2), 7.07-7.11 (2H, m, Ar-H), 7.54-7.58 (2H, m, Ar-H), 8.17 (1H, d, *J* = 2 Hz, Ar-H), 8.71 (1H, d, *J* = 2 Hz, Ar-H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ 51.76 (OMe), 85.83 (C), 92.75 (C), 100.89 (2-C), 115.88 (d, *J* = 21 Hz, 3' and 5'-CH), 118.43 (d, *J* = 3 Hz, C), 118.92 (C), 133.42 (CH), 133.70 (d, *J* = 10 Hz, 2' and 6'-CH), 133.76 (C), 145.07 (C), 147.29 (C), 148.69 (CH), 162.92 (d, *J* = 252 Hz, C-F), 165.17 (C=O) ppm. Elemental Analysis calcd. for $C_{17}H_{11}FN_2O_2S$ C, 62.57; H, 3.40; N, 8.58; S, 9.83%. Found C, 62.25; H, 3.53; N, 8.24; S, 9.99%.

4.1.1.11. Methyl 3-amino-6-[2-(pyridin-2-yl)ethynyl]thieno[3,2-*b*]pyridine-2-carboxylate (2k): Yellow solid (117 mg, 70%), m.p. 226-228 ºC. ^{1}H NMR (DMSO-d_6, T = 60 ºC, 300 MHz) δ 3.84 (3H, s, OMe), 6.81 (2H, br s, NH_2), 7.42-7.47 (1H, m, Ar-H), 7.70 (1H, d, *J* = 7.8 Hz, Ar-H), 7.85-7.91 (1H, m, Ar-H), 8.63-8.66 (2H m, Ar-H), 8.84 (1H, d, *J* = 2 Hz, Ar-H) ppm. ^{13}C NMR (DMSO-d_6, T = 60 ºC, 75.4 MHz) δ 51.33 (OMe), 85.03 (C), 92.47 (C), 99.06 (2-C), 117.04 (C), 123.66 (CH), 127.33 (CH), 132.83 (C), 134.32 (CH), 136.54 (CH), 141.52 (C), 145.33 (C), 147.22 (C), 148.43 (C), 150.01 (CH), 164.08 (C=O) ppm. MS (EI) m/z 309 (M^+, 100), 277 (63). HRMS calcd. for $C_{16}H_{11}N_3O_2S$ M^+ 309.0572; found 309.0571.

4.1.1.12. Methyl 3-amino-6-[2-(pyridin-3-yl)ethynyl]thieno[3,2-*b*]pyridine-2-carboxylate (2l): Yellow solid (150 mg, 90%), m.p. 205-207 ºC. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.93 (3H, s, OMe), 6.22 (2H, br s, NH_2), 7.36-7.38 (1H, m, Ar-H), 7.44-7.50 (1H, m, Ar-H), 7.64-7.70 (1H, m, Ar-H), 7.87 (1H, d, *J* = 8 Hz, Ar-H), 8.21 (1H,d, *J* = 2 Hz, Ar-H), 8.74 (1H, d, *J* = 2 Hz, Ar-H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.78 (OMe), 89.52 (C), 90.08 (C), 101.18 (2-C), 118.18 (C), 119.78 (C), 123.29 (CH), 128.55 (C), 132.02 (CH), 133.61 (C), 133.65 (CH), 138.78 (CH), 145.54 (C), 147.27 (C), 148.74 (CH), 165.13 (C=O) ppm. MS (EI) m/z 309 (M^+, 53), 277 (100). HRMS calcd. for $C_{16}H_{11}N_3O_2S$ M^+ 309.0572; found 309.0578.

4.1.1.13. Methyl 3-amino-6-[2-(thien-3-yl)ethynyl)]thieno[3,2-*b*]pyridine-2-carboxylate (2m): Yellow solid (119 mg, 70%), m.p. 165-167 ºC. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.93 (3H, s, OMe), 6.22 (2H, br s, NH_2), 7.24 (1H, dd, *J* = 4.8 and 1.2 Hz, Ar-H), 7.35 (1H, dd, *J* = 4.8 and 2.8 Hz, Ar-H), 7.62 (1H, dd, *J* = 2.8 and 1.2 Hz, Ar-H), 8.16 (1H, d, *J* = 2 Hz, Ar-H), 8.71 (1H, d, *J* = 2 Hz, Ar-H) ppm. ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 51.74 (OMe), 85.72 (C), 89.05 (C), 100.78 (2-C), 119.09 (C), 121.40 (C), 125.76 (CH), 129.74 (2 × CH), 133.32 (CH), 133.70 (C), 144.97 (C), 147.32 (C), 148.68 (CH), 165.18 (C=O) ppm. MS (EI) m/z 314 (M^+, 100), 282 (90). HRMS calcd. for $C_{15}H_{10}N_2O_2S_2$ M^+ 314.0184; found 314.0183.

4.2 Biological activity

4.2.1. Material and Methods

4.2.1.1. Reagents

Fetal bovine serum (FBS), L-glutamine, phosphate buffered saline (PBS) and trypsin were from Gibco Invitrogen Co. (Scotland, UK). RPMI-1640 medium was from Cambrex (New Jersey, USA). Acetic acid, dimethyl sulfoxide (DMSO), doxorubicin, penicillin, streptomycin, ethylenediaminetetraacetic acid (EDTA), sulforhodamine B (SRB) and trypan blue were from SigmaChemical Co. (Saint Louis, USA). Tricloroacetic acid (TCA) and Tris were sourced from Merck (Darmstadt, Germany).

4.2.1.2. Solutions of the compounds
Stock solutions of the tested compounds were prepared in DMSO and kept at −70°C. Appropriate dilutions were freshly prepared in the test medium just prior to the assays. The effect of the vehicle solvent (DMSO) on the growth of the cell lines was evaluated by exposing untreated control cells to the maximum concentration of DMSO used in the assays (0.25%). No influence was found (data not shown).

4.2.1.3. Cell cultures
Three human tumor cell lines, MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and A375-C5 (melanoma) were used. MCF-7 and A375-C5 were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK), and NCI-H460 was kindly provided by the National Cancer Institute (NCI, Bethesda, USA). They were routinely maintained as adherent cell cultures in RPMI-1640 medium supplemented with 5% heat-inactivated FBS, 2 mM glutamine and antibiotics (penicillin 100 U/mL, streptomycin 100 μg/mL), at 37°C in a humidified atmosphere containing 5% CO_2. Exponentially growing cells were obtained by plating 1.5×10^5cells/mL for MCF-7 and 0.75×10^5cells/mL for A375-C5 and NCI-H460, followed by a 24-h incubation.

4.2.1.4. Growth inhibition assay
The effects on the *in vitro* growth of human tumor cell lines were evaluated according to the procedure adopted by the NCI (USA) in their "*In vitro* Anticancer Drug Discovery Screen", using the protein-binding dye sulforhodamine B to assess cell growth.[8,9] Briefly, exponentially growing cells were exposed for 48 h, in 96-well microtiter plates, to five serial dilutions of each test compound, starting from a maximum concentration of 150 μM (if possible). Following the exposure period adherent cells were fixed *in situ* with TCA, washed and stained with SRB. The bound stain was solubilized and the absorbance was measured at 492 nm in a plate reader (Bio-tek Instruments Inc., Powerwave XS, Wincoski, USA). Dose-response curves were obtained for each test compound and cell line, and the growth inhibition of 50% (GI_{50}), corresponding to the concentration of the compounds that inhibited 50% of the net cell growth was calculated as described elsewhere.[9] Doxorubicin was tested in the same manner, to be used as a positive control.

4.2.1.5. Flow cytometric assays for cell cycle distribution analysys and apoptosis detection with annexin V-FITC/PI assay
NCI-H460 cells were plated at 0.75×10^5cells/mL in 6-well plates and left incubating for 24 h. Cells were then incubated with complete medium only (control), medium with the compound's solvent (DMSO) or with compounds **2d, 2e** and **2g** at their respective GI_{50} concentrations previously obtained by the SRB assay (0.32, 5.7 and 8.9 μM). Cells were harvested following a 48 h incubation with the compounds and further processed for either cell cycle analysis or apoptosis detection. For cell cycle analysis, cells were fixed in 70 % ethanol and subsequently resuspended in PBS containing 0.1 mg/mL RNase A and 5 μg/mL propidium iodide, prior to analysis. Induced apoptosis was assayed by the Human Annexin V-FITC/PI apoptosis Kit (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions.[10]
Cellular DNA content (for cell cycle distribution analysis and presence of sub-G1 peak, suggestive of apoptosis induction) and measurement of phosphatidylserine externalization were analyzed using an Epics XL-MCL Coulter flow cytometer plotting at least 20,000 events per sample. Cell cycle distribution and apoptosis data analysis was subsequently performed using FlowJo 7.2 software (Tree Star, Inc.). Experiments were performed in duplicate in three to six independent occasions and the average and standard error of the obtained results were presented. Statistical analysis was performed for the induction of apoptosis results by the non-parametric Friedman's test followed by Dunn's Post-test using GraphPad Prism 5 software. P values < 0.05 were considered as statistically significant.

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